Supporting Information

Short title

A. thaliana **inositol pyrophosphate phosphohydrolases**

Article title

Arabidopsis PFA-DSP-type phosphohydrolases target specific inositol pyrophosphate messengers

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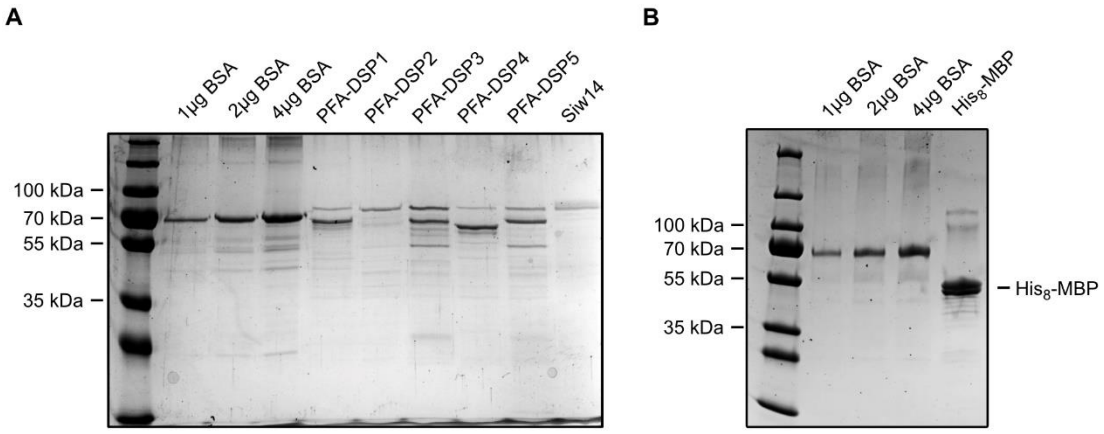

Figure S1: Purification of PFA-DSP proteins. (A, B) Recombinant His-MBP-PFA-DSPs or His-MBP-Siw14 were expressed in *E. coli* and purified with Ni-NTA resin as described in methods. Dialyzed proteins were denatured and separated by SDS-PAGE in parallel with BSA standards to determine protein concentrations by staining with Coomassie blue.

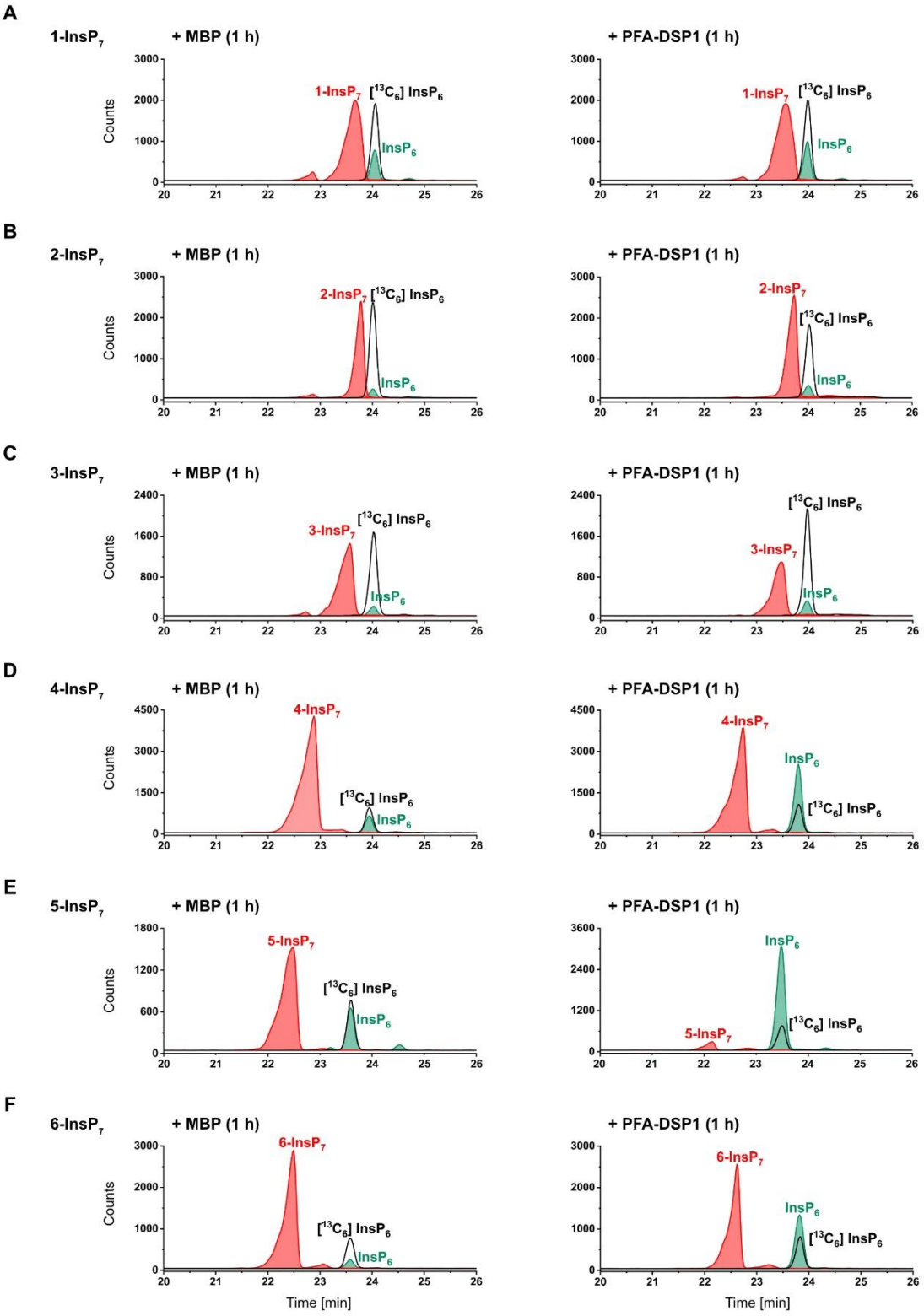

Figure S2: *In vitro*, *Arabidopsis* PFA-DSP1 displays robust PP-InsP phosphohydrolase activity against 5-InsP$_7$ and partial phosphohydrolase activity against 4-InsP$_7$ and 6-InsP$_7$, respectively. (A – F) 0.4 µM PFA-DSP1 was incubated with 0.33 mM InsP$_7$ and 1 mM $MgCl_2$ for 1 h. The reaction product was spiked with an isotopic standards mixture ([$^{13}C_6$]1,5-InsP$_8$, [$^{13}C_6$]5-InsP$_7$, [$^{13}C_6$]1-InsP$_7$, [$^{13}C_6$] InsP$_6$, [$^{13}C_6$]2-OH InsP$_5$) and subjected to CE-ESI-MS analyses. Representative extracted-ion electropherograms of samples shown in Figure 1.

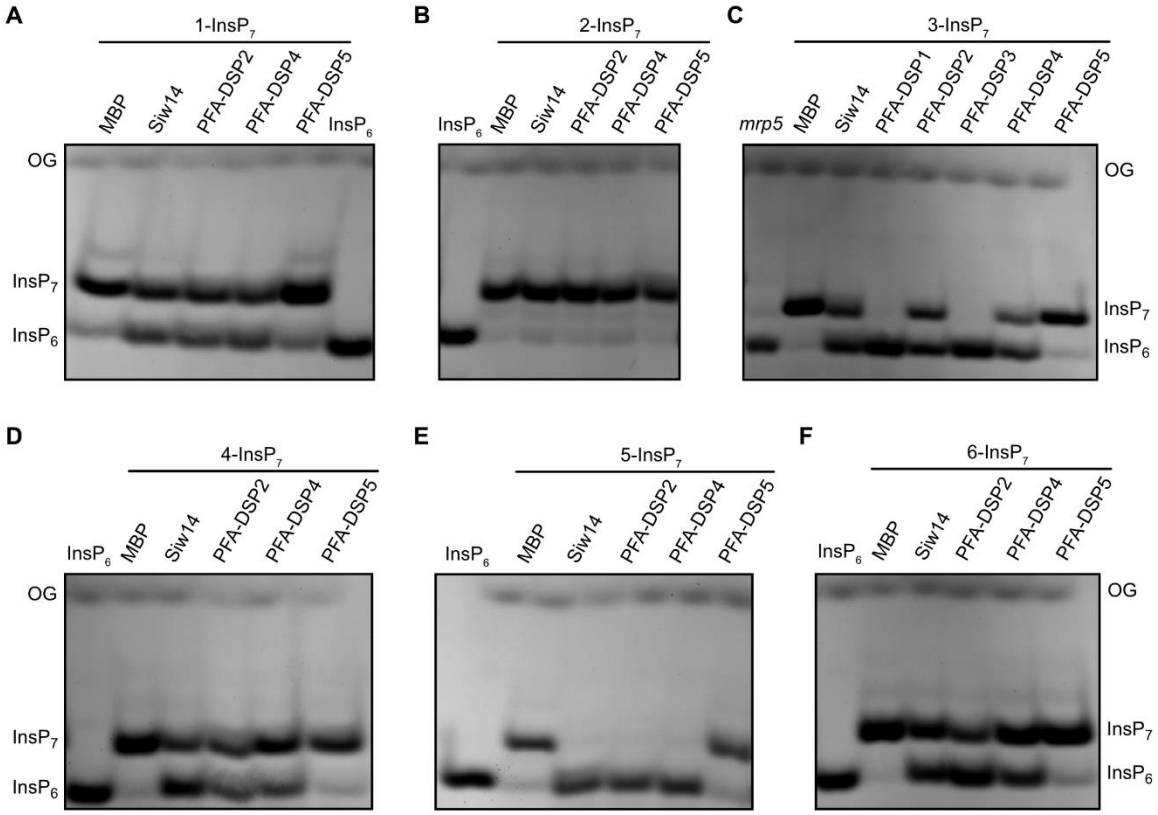

Figure S3: In the absence of divalent cations, all $InsP_7$ isomers with the exception of 2-$InsP_7$ become substrates for selected *Arabidopsis* PFA-DSPs *in vitro*. (A – F) Approximately 0.4 µM His-MBP-PFA-DSPs and His-MBP were incubated with 1 mM EDTA and 0.33 mM $InsP_7$ for 1 h at 22°C. His-MBP served as a negative control. The reaction products were separated by 33 % PAGE and visualized with toluidine blue. The identity of bands was determined by migration compared to $InsP_6$ or (C) compared to TiO_2-purified *mrp5* seed extract.

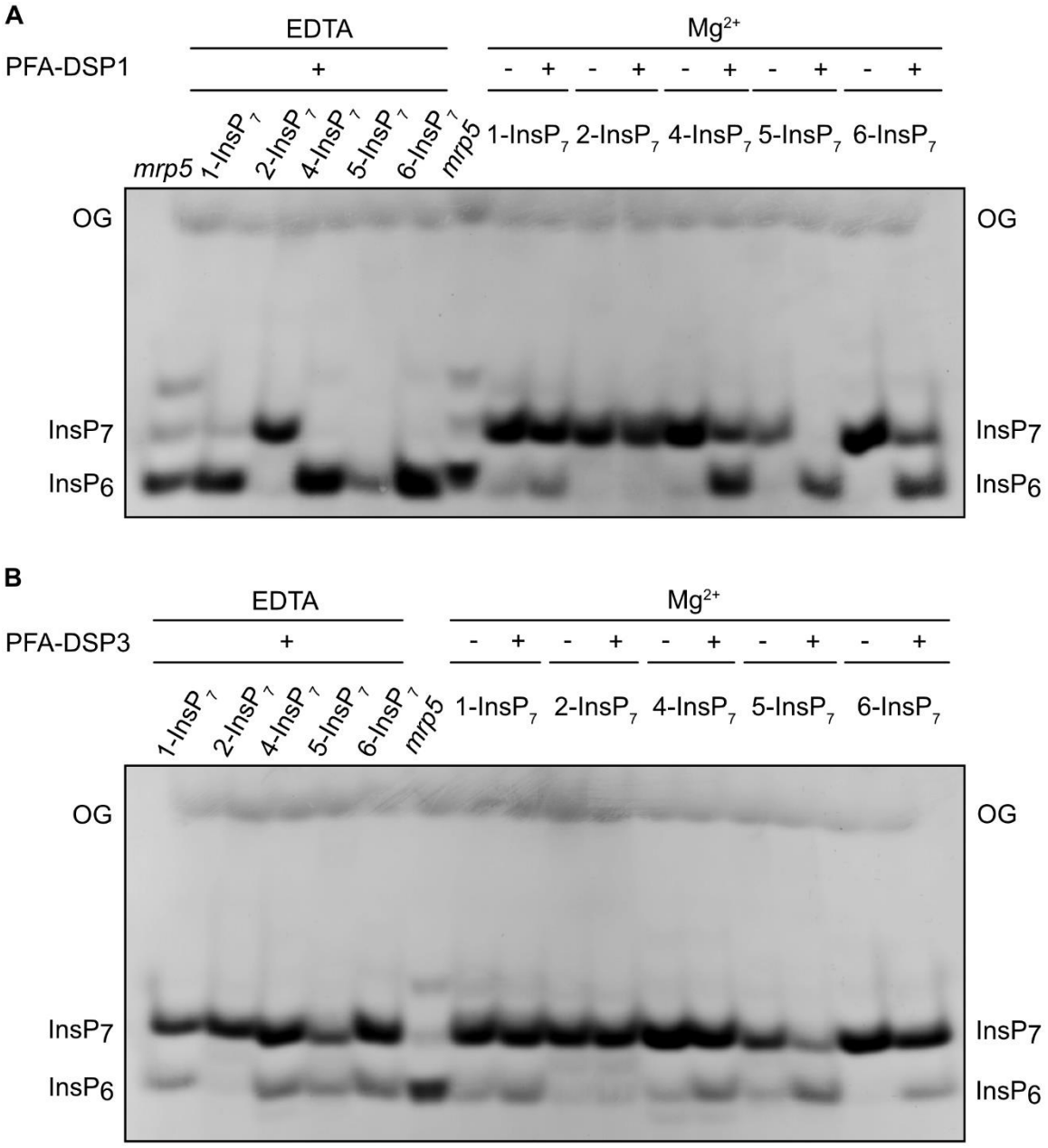

Figure S4: In the presence of Mg^{2+}, PFA-DSP1 and PFA-DSP3 display robust *in vitro* $InsP_7$ phosphohydrolase activity with high specificity for the 5-β-phosphate. (A – B) Approximately 0.4 µM His-MBP-PFA-DSP1 and His-MBP-PFA-DSP3 were incubated with 0.33 mM $InsP_7$ and 1 mM EDTA or 1 mM $MgCl_2$ for 1 h at 22°C. His-MBP served as a negative control. The reaction products were separated by 33 % PAGE and visualized with toluidine blue. The identity of bands was determined by migration compared to TiO_2-purified *mrp5* seed extract.

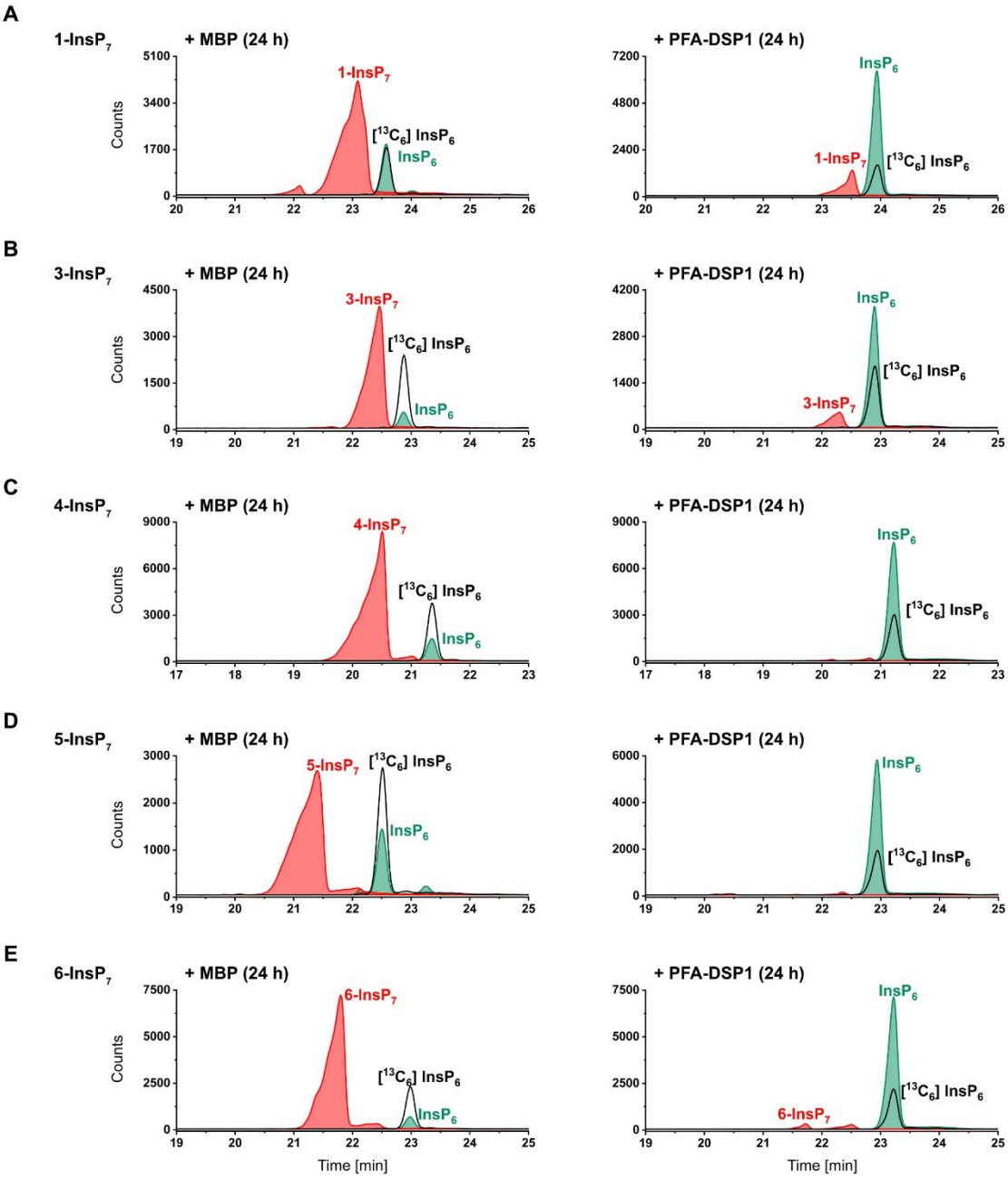

Figure S5: Under prolonged incubation time, *Arabidopsis* PFA-DSP1 efficiently hydrolyzes 5-InsP$_7$, 4-InsP$_7$ and 6-InsP$_7$ but only displays partial activities against 1-InsP$_7$ and 3-InsP$_7$. (A – E) 0.4 µM PFA-DSP1 was incubated with 0.33 mM InsP$_7$ and 1 mM $MgCl_2$ for 24 h. The reaction product was spiked with an isotopic standards mixture ([$^{13}C_6$]1,5-InsP$_8$, [$^{13}C_6$]5-InsP$_7$, [$^{13}C_6$]1-InsP$_7$, [$^{13}C_6$] InsP$_6$, [$^{13}C_6$]2-OH InsP$_5$) and subjected to CE-ESI-MS analyses. Representative extracted-ion electropherograms of samples shown in Figure 2.

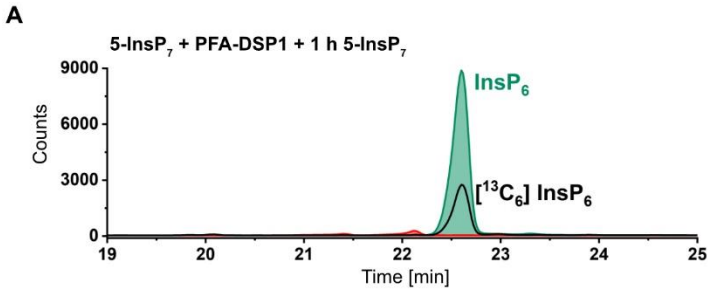

Figure S6: *Arabidopsis* PFA-DSP1 maintains 5-InsP$_7$ phosphohydrolase activity during prolonged incubation time *in vitro*. (A) 0.4 µM PFA-DSP1 was incubated with 0.33 mM 5-InsP$_7$ and 1 mM $MgCl_2$ for 24 h. To ensure that PFA-DSP1 is active during the whole incubation time, 0.33 mM 5-InsP$_7$ was added after 23 h and incubated for another 1 h. The reaction product was spiked with an isotopic standards mixture ([$^{13}C_6$]1,5-InsP$_8$, [$^{13}C_6$]5-InsP$_7$, [$^{13}C_6$]1-InsP$_7$, [$^{13}C_6$] InsP$_6$, [$^{13}C_6$]2-OH InsP$_5$) and subjected to CE-ESI-MS analyses.

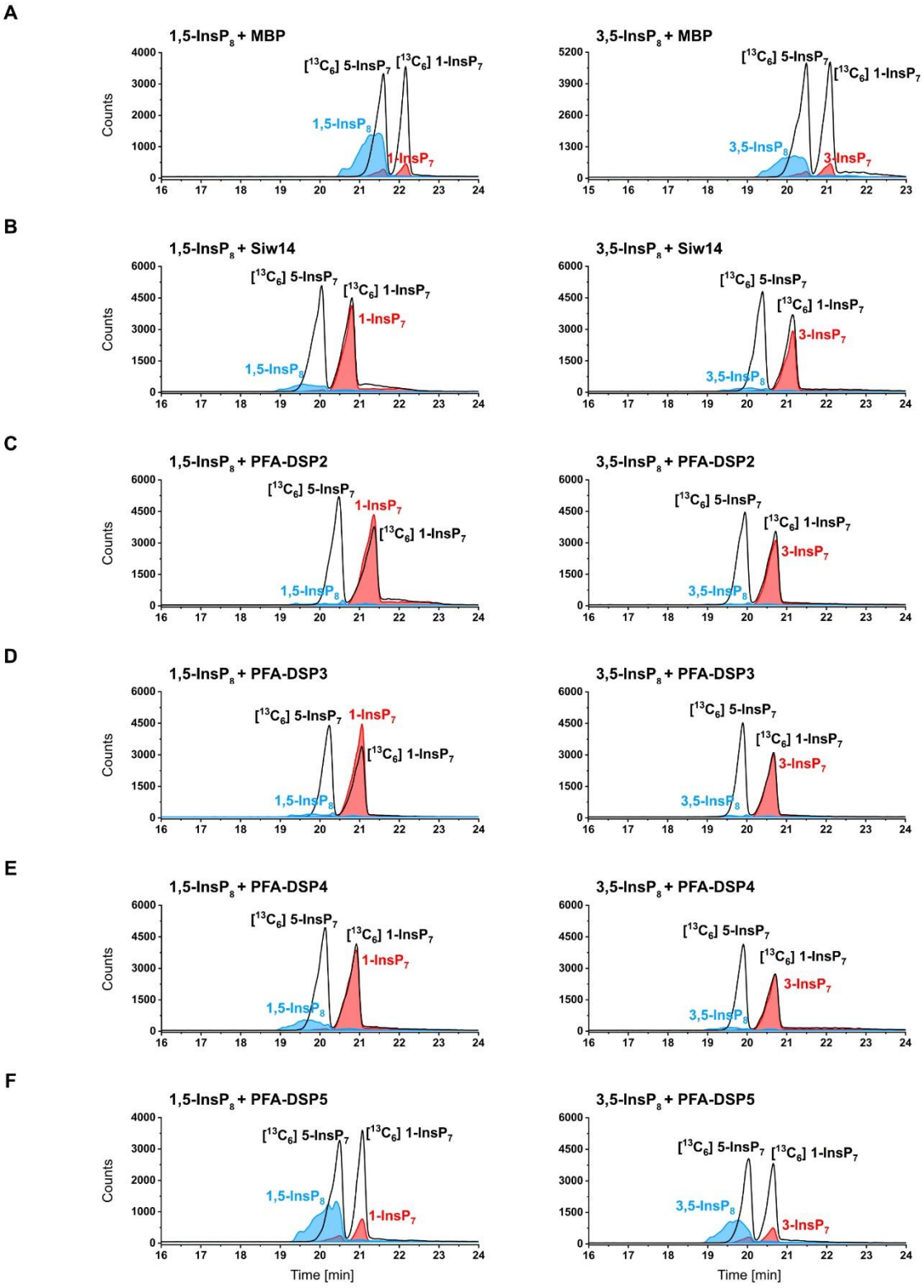

Figure S7: *In vitro*, *Arabidopsis* PFA-DSPs display robust 1/3,5-InsP$_8$ phosphohydrolase activity. (A – F) Approximately 0.4 µM His-MBP-PFA-DSPs and His-MBP-Siw14 were incubated with 0.33 mM 1,5-InsP$_8$ or 3,5-InsP$_8$ and 1 mM $MgCl_2$ for 1 h. The reaction products were spiked with isotopic standards mixture ([$^{13}C_6$]1,5-InsP$_8$, [$^{13}C_6$]5-InsP$_7$, [$^{13}C_6$]1-InsP$_7$, [$^{13}C_6$] InsP$_6$, [$^{13}C_6$]2-OH InsP$_5$) and subjected to CE-ESI-MS analyses. Representative extracted-ion electropherograms of samples shown in Figure 3C.

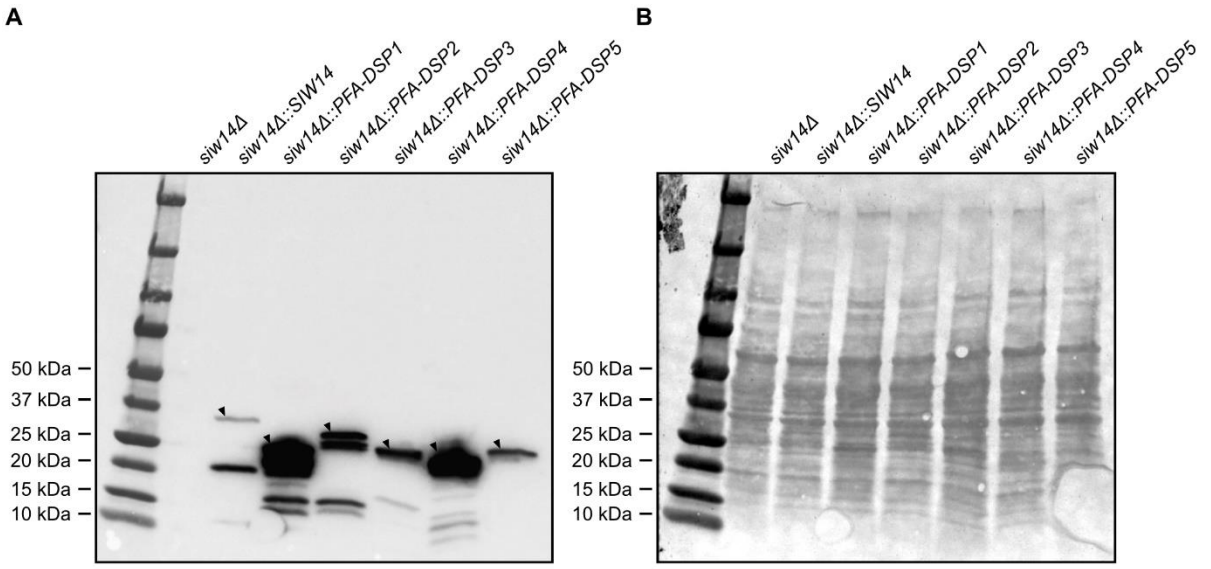

Figure S8: All five PFA-DSP homologs are stably expressed in the *siw14Δ* yeast strain. Immunoblot analyses of protein extracts from *siw14Δ* yeast transformed with either empty pDRf1-GW plasmid or pDRf1-GW carrying *SIW14* or *PFA-DSP1–5* encoding translational fusions with a C-terminal V5-tag. (A) For detection of V5-tagged proteins, an anti-V5 tag primary antibody (Invitrogen; 1:2000 dilution) and an anti-mouse secondary antibody coupled with HRP (Bio-Rad; goat; 1:10000 dilution) were used. The chemiluminescence signal of the ECL substrate (Bio-Rad) was detected using the ChemiDoc MP imager (Bio-Rad). Black arrows indicate the specific protein bands based on the calculated molecular weight. (B) Ponceau staining of the same blot.

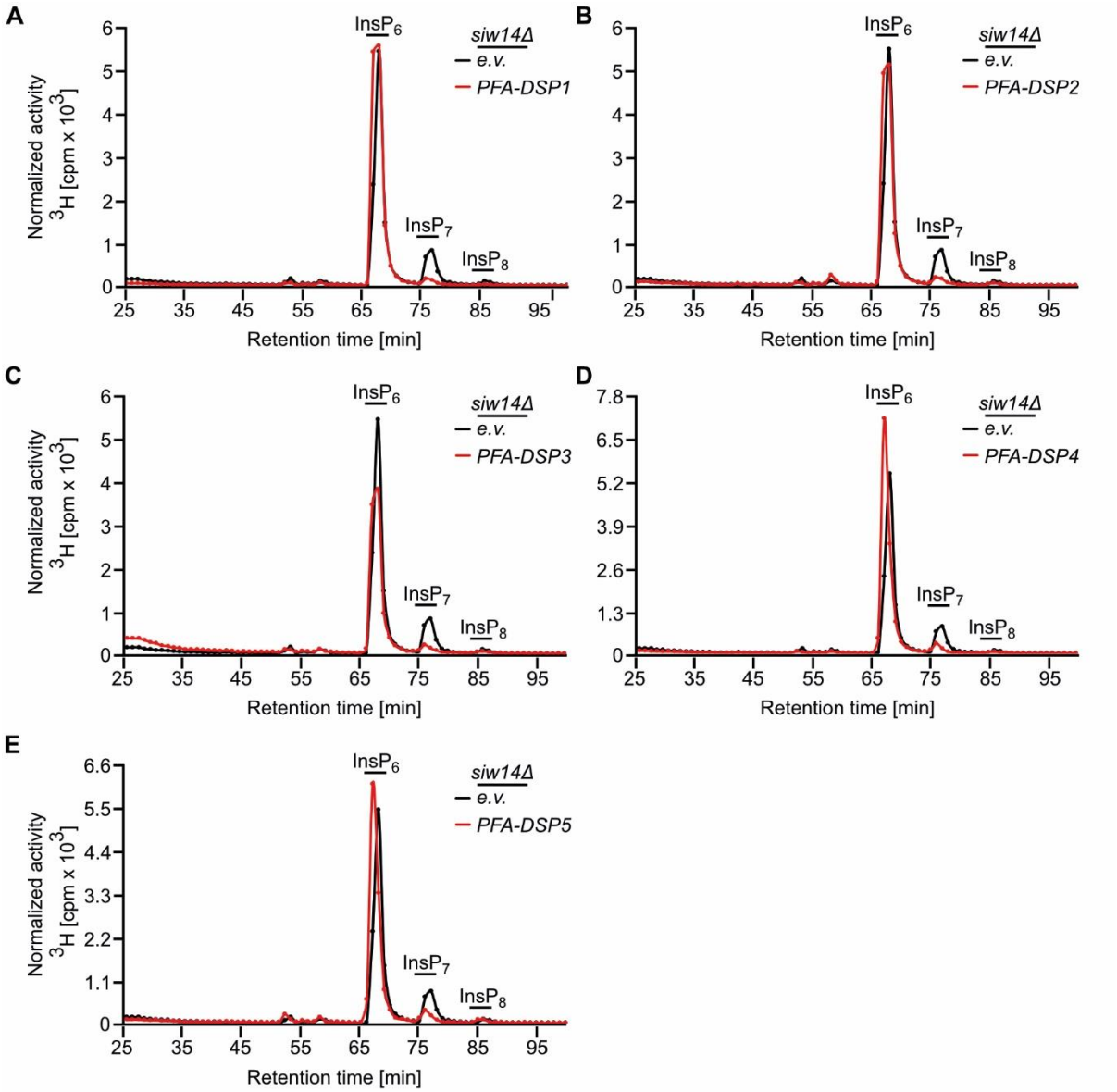

Figure S9: Heterologous expression of *Arabidopsis* PFA-DSPs complements *siw14Δ*-associated defects in $InsP_7/InsP_6$ ratios in yeast. (A - E) SAX-HPLC profiles of radiolabeled *siw14Δ* yeast transformed with either empty pDRf1-GW plasmid (e.v.) or pDRf1-GW carrying *PFA-DSP1 - 5*. Depicted is a representative analysis of each *PFA-DSP* transformant, with the same analysis of a representative empty vector transformant shown in the same profile in each graph. The experiment was repeated twice (n = 3) with similar results (combined data shown in Figure 4B).

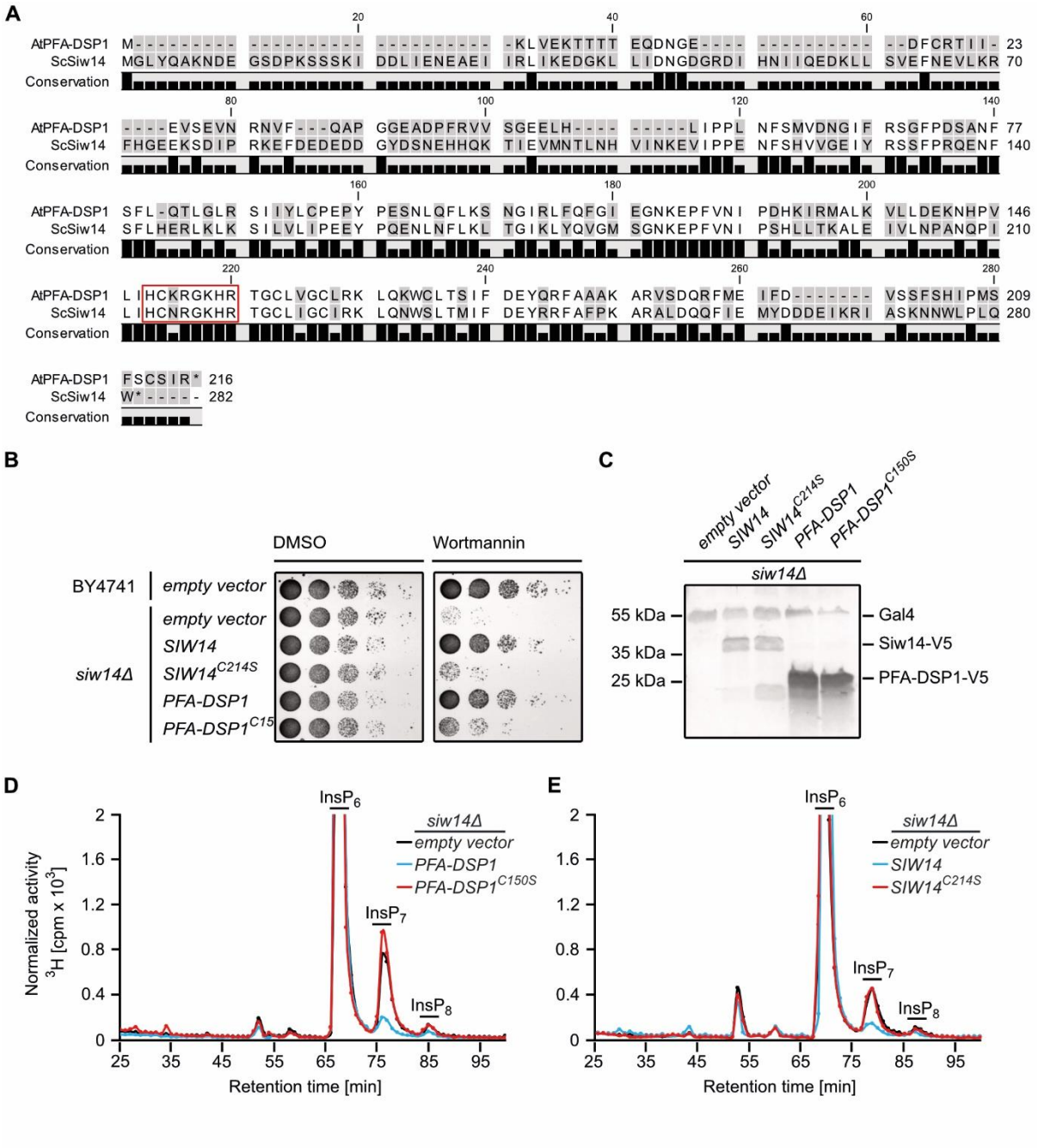

Figure S10: Complementation of *siw14Δ*-associated growth defects depends on catalytic activity. (A) Protein alignment of Siw14 from yeast and its homolog PFA-DSP1 from *Arabidopsis thaliana*. Identical amino acids are shown in black, different residues are highlighted with grey boxes. The conserved PTP (Protein Tyrosine Phosphatase) signature motif HC(X)5R is highlighted with the red box. The alignment was generated via the Multiple Alignments function of CLC Main Workbench 8 (QIAGEN). (B) Growth complementation assay with *siw14Δ*. Wild-type yeast (BY4741) and the *siw14Δ* yeast mutant were transformed with pDRf1-GW plasmids carrying either *SIW14* or its catalytic mutant C214S or carrying *PFA-DSP1* or its catalytic mutant C150S. Yeast strains transformed with empty pDRf1-GW vector as indicated served as controls. Transformants were then spotted in 8-fold serial dilutions (starting from OD 1.0) onto selective media containing wortmannin solved in DMSO or DMSO alone as control. Plates were incubated at 26 °C for 2 days before photographing. (C) Immunoblotting of Siw14, Siw14^{C214S}, PFA-DSP1 and PFA-DSP1^{C150S}. For detection of V5-tagged proteins an anti-V5 tag primary antibody

(Invitrogen; 1:2000 dilution) and an anti-mouse secondary antibody coupled with Alexa Fluor plus 800 (Invitrogen; goat; 1:20000 dilution) were used. As loading control, Gal4 protein levels were detected simultaneously using a polyclonal anti-Gal4 antibody (Santa Cruz; 1:1000 dilution) and an anti-rabbit StarBright Blue 700 antibody (Bio-Rad, goat; 1:2500 dilution). The signal was detected using the multiplex function of the ChemiDoc MP imager (Bio-Rad). (D) SAX-HPLC profiles of extracts of radiolabeled *siw14Δ* yeast transformed with either empty pDRf1-GW plasmid (empty vector) or pDRf1-GW carrying either *SIW14* or *SIW14*^{C214S}. (E) SAX-HPLC profiles of radiolabeled *siw14Δ* yeast transformed with either empty pDRf1-GW plasmid (e.v.) or pDRf1-GW carrying either *PFA-DSP1* or *PFA-DSP1*^{C150S}. (B – E) The experiments were repeated independently with similar results.

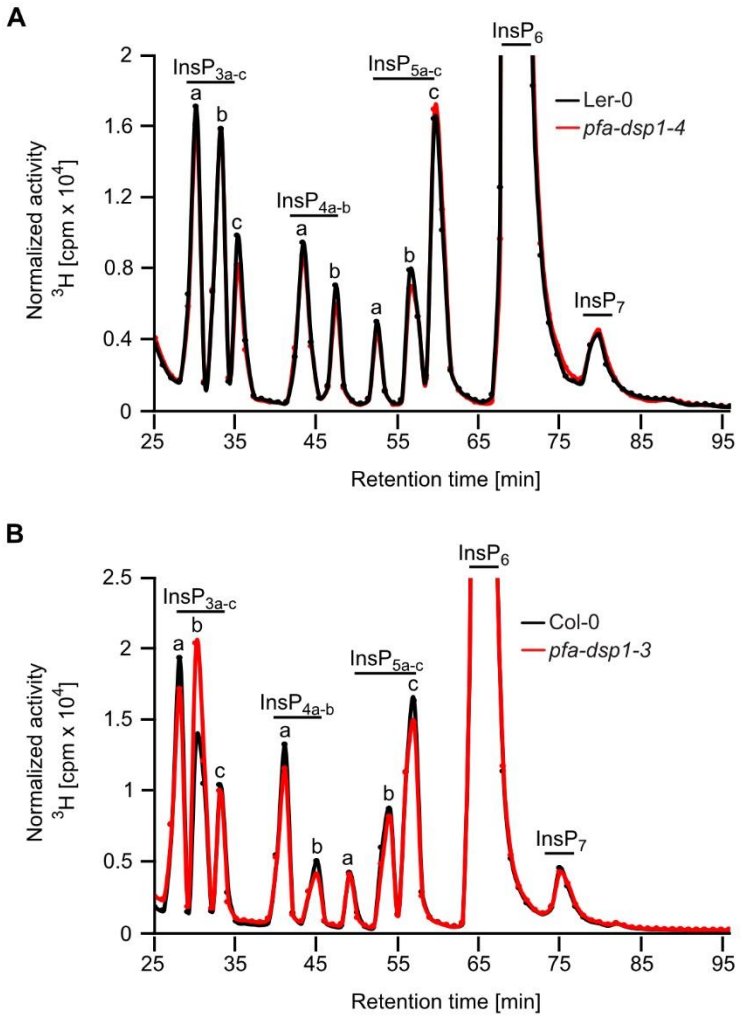

Figure S11: Single mutant *Arabidopsis pfa-dsp1* loss-of-function lines do not display InsP/PP-InsP defects. Representative SAX-HPLC profiles of 20-days-old wild-type Ler-0 and *pfa-dsp1-4 Arabidopsis* seedlings (A) and of Col-0 and *pfa-dsp1-3 Arabidopsis* seedlings (B) radiolabeled with [^{3}H]-*myo*-inositol. All visible peaks are highlighted and assigned to the corresponding InsP species. Based on published chromatographic mobilities [1, 2], $InsP_{4a}$ likely represents Ins(1,4,5,6)P_4 or Ins(3,4,5,6)P_4, $InsP_{5a}$ likely represents $InsP_5$ [2-OH], $InsP_{5b}$ likely represents $InsP_5$ [4-OH] or its enantiomeric form $InsP_5$ [6-OH], and $InsP_{5c}$ likely represents $InsP_5$ [1-OH] or its enantiomeric form $InsP_5$ [3-OH]. The isomeric natures of $InsP_{3a-c}$, $InsP_{4b}$, $InsP_7$, and $InsP_8$ are unknown.

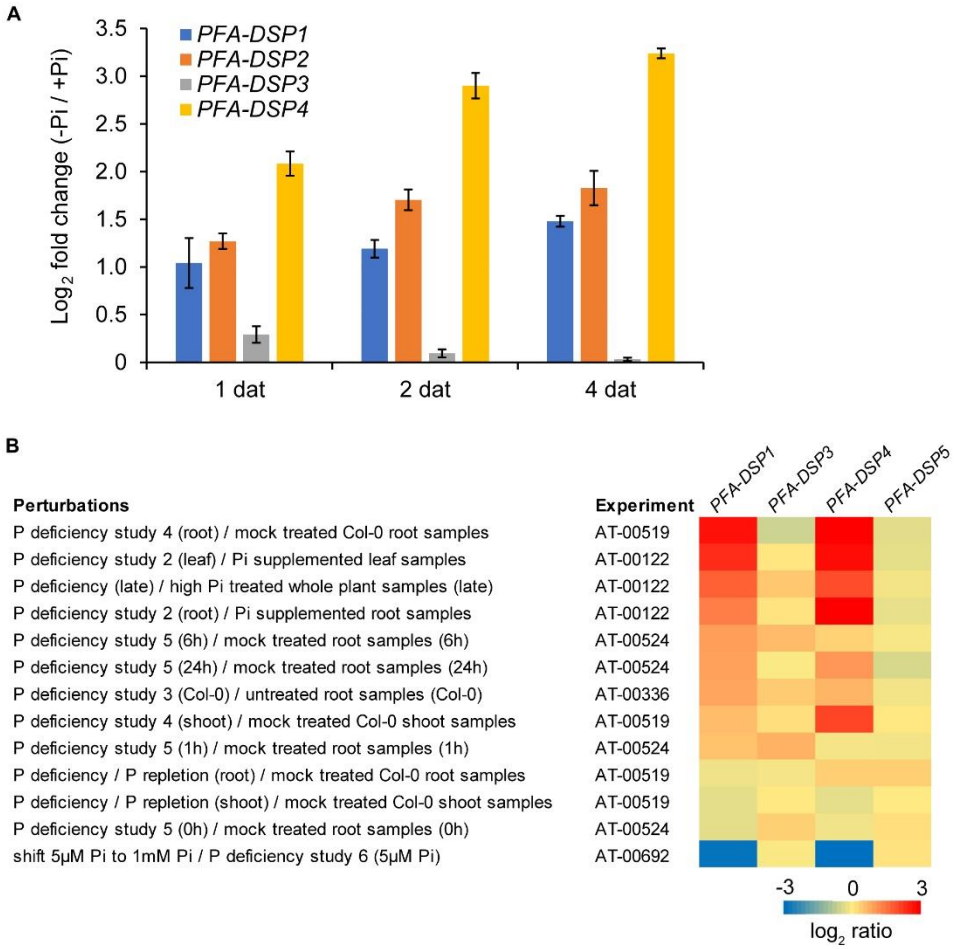

Figure S12: *Arabidopsis PFA-DSP1, 2* and *4* are strongly induced by P_i deficiency. (A) Expression of the indicated *PFA-DSP*s in roots of *Aradidopsis thaliana* (accession Col-0) plants according to a transcriptome experiment with Agilent microarrays [3]; data deposited on e!DAL repository under the accession code https://doi.org/10.5447/IPK/2018/4. No probe for *PFA-DSP5* was present in the microarray chips. Seven-day-old plants pre-cultured on sufficient P_i supply were transferred to fresh solid media containing 625 µM P_i (+Pi) or 100 µM P_i (-Pi). Whole roots were collected at the indicated time points after transfer. Data represent means ± SD (n = 3). (B) Heatmap analysis of *PFA-DSP*s genes in response to the indicated P_i treatmens. No data are presented for *PFA-DSP2* as no probe for this gene is present in Affimetrix chips. Transcriptional data were retrieved and analyzed with Genevestigator (http://www.genevestigator.ethz.ch).

Table S1: Overview of *Arabidopsis* PFA-DSP substrate specificities in presence of Mg^{2+} showing a robust PP-InsP phosphohydrolase activity against 5-InsP_7, 1,5-InsP_8 and 3,5-InsP_8, *in vitro*. The table summarizes the *in vitro* results of Figure 1, 3, S2 and S7. (-) indicates no substrate, (+) poor substrate, (++) good substrate and n.d. no data.

Mg^{2+}	1-InsP_7	2-InsP_7	3-InsP_7	4-InsP_7	5-InsP_7	6-InsP_7	1,5-InsP_8	3,5-InsP_8
Siw14	(-)	(-)	(-)	(+)	(++)	(+)	(++)	(++)
PFA-DSP1	(+)	(-)	(+)	(+)	(++)	(+)	(++)	(++)
PFA-DSP2	(-)	(-)	(-)	(+)	(++)	(+)	(++)	(++)
PFA-DSP3	(+)	(-)	(+)	(+)	(++)	(+)	(++)	(++)
PFA-DSP4	(+)	(-)	(+)	(+)	(++)	(+)	(++)	(++)
PFA-DSP5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
PFA-DSP5 *	(-)	(-)	(-)	(+)	(++)	(+)	n.d.	n.d.

* tested with a higher PFA-DSP5 concentration and increased incubation time

Table S2: Oligonucleotide sequences.

Primer name	Sequence
attB1 adapter	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC
attB2 adapter	GGGGACCACTTTGTACAAGAAAGCTGGGTC
attB2+V5 adapter	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACGTAGAATCGAGACCGAGGAGAGGG TTAGGGATAGGCTTACCTCCTCCAGATCC
attB1_ScSIW14	AAAAAGCAGGCTTCATGGGTTTATATCAAGCAAAG
attB2_ScSIW14s	AAAAAGCAGGCTTCATGGGTTTATATCAAGCAAAG
attB2_ScSIW14V5	CTTACCTCCTCCAGATCCCCATTGTAGAGGCAACCAG
attB1_AtPFA-DSP1	AAAAAGCAGGCTTCATGAAGCTTGTGGAGAAGAC
attB2_AtPFA-DSP1ns	AGAAAGCTGGGTCCCTGATGGAACAAGAGAATG
attB2_AtPFA-DSP1s	AGAAAGCTGGGTCTTACCTGATGGAACAAGAG
attB2_AtPFA-DSP1V5	ACCTCCTCCAGATCCCCTGATGGAACAAGAGAATG
attB1_AtPFA-DSP2	AAAAAGCAGGCTTCATGAAACTGATTGAGAAGACG
attB2_AtPFA-DSP2s	AGAAAGCTGGGTCTTACCTATTGGAGCAAGAAAAAG
attB2_AtPFA-DSP2V5	ACCTCCTCCAGATCCCCTATTGGAGCAAGAAAAAGAC
attB1_AtPFA-DSP3	AAAAAGCAGGCTTCATGTGTTTGATTATGGAAACGG
attB2_AtPFA-DSP3s	AGAAAGCTGGGTCTTAAACTCTAGCAGCCTGCG
attB2_AtPFA-DSP3V5	ACCTCCTCCAGATCCAACTCTAGCAGCCTGCGG
attB1_AtPFA-DSP4	AAAAAGCAGGCTTCATGACGTTAGAGAGTTACGCCG
attB2_AtPFA-DSP4s	AGAAAGCTGGGTCTCAGTAATCAATAGTATTAGTATACCTCTTGG
attB2_AtPFA-DSP4V5	ACCTCCTCCAGATCCGTAATCAATAGTATTAGTATACCTCTTGG
attB1_AtPFA-DSP5	AAAAAGCAGGCTTCATGGGCTTAATTGTGGATGATG
attB2_AtPFA-DSP5s	AGAAAGCTGGGTCTTATCCTTTGGTGGCTTGAGG
attB2_AtPFA-DSP5V5	ACCTCCTCCAGATCCTCCTTTGGTGGCTTGAGG
ScSIW14_C214S_F	TCAACCGATACTGATACATTCTAATAGAGGCAAACATAGAAC
ScSIW14_C214S_R	GTTCTATGTTTGCCTCTATTAGAATGTATCAGTATCGGTTGA
AtPFA-DSP1_C150S_F	GTTCTGATTCATAGTAAGCGAGGC
AtPFA-DSP1_C150S_R	GCCTCGCTTACTATGAATCAGAACA
ScSIW14pgt_PstI_F	AGCCTGCAGGATGGAGCTGCTCCTGGCTG
ScSIW14pgt_EcoRI_R	GAATTCAATATAAAGCGGGAATTTTTTTTTTTC
AtPFA-DSP1_267_F	ATACTTGTGCCCGGAGCCCT
AtPFA-DSP1_373_R	TCACAAATGGCTCCTTGTTGCCT
AtTIP41-like_F	TGGTTGGAAGCAGGAAGGGCT
AtTIP41-like_R	TGCTGAGACGGCTTGCTCCTGA
AtPP2AA3_F_qPCR	TGGTGCTCAGATGAGGGAGA
AtPP2AA3_R_qPCR	TAGCACATCTGGGGCACTTG
ScSIW14_pUG_F	CTCTTCTGGATCAATTTTTCTTTTTCATCTAAAGTTTAAAAGGAGCAGCTGAAGCTTCGTA CGC
ScSIW14_pUG_R	CATCATTTTCGAAGAGACTAGTTACGTAAAGGTAATCACTGTCTACATAGCATAGGCCAC TAGTGGATCTG
WiscDsLox_473B10_LP	TTGTTTTGCAAAACTGCAAAG
WiscDsLox_473B10_RP	TTGCCTTCAATACCAAACTGG
P745_WiscDsLox_F	AACGTCCGCAATGTGTTATTAAGTTGTC
GT1415_F	CGACTCTCCTCACCTAAAGATTCA
GT1415_R	GTTGCCTTCAATACCAAACTGG
DS3-1	ACCCGACCGGATCGTATCGGT
SAIL_116_C12_LP	TTGTTTTGCAAAACTGCAAAG
SAIL_116_C12_RP	TTGCCTTCAATACCAAACTGG
LB1_SAIL_F	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC

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References

1. Kuo, H. F., Hsu, Y. Y., Lin, W. C., Chen, K. Y., Munnik, T., Brearley, C. A., and Chiou, T. J. (2018) Arabidopsis inositol phosphate kinases IPK1 and ITPK1 constitute a metabolic pathway in maintaining phosphate homeostasis, Plant J.
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